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Embryonic Coat of the Grass Shrimp *Palaemonetes pugio*

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Abstract. The embryo of the grass shrimp, *Palaemonetes pugio,* is surrounded during development by a protective extracellular coat designated as the embryonic coat (EC). At hatching, this EC is composed of four embryonic envelopes (EE), each of which is composed of multiple layers. The outermost layer of the EC, the outer investment coat (OIC), is derived primarily, if not completely, from pleopods of the female. The first envelope (EE1) forms as a bilayered envelope, $EE1_a$ and $EE1_b$, immediately after oviposition. The OIC becomes closely associated with EE1 and remains in close contact with EE1 until hatching occurs. An additional layer, $EE1_c$, is added to the inner side of EE1 between 3 and 5 d after oviposition. Three more embryonic envelopes, EE2, EE3, and EE4, are formed between the embryo and EE1 by 7 d after oviposition. Formation of embryonic envelopes continues until 10 d after oviposition; by this time each envelope is morphologically distinct in composition, with "outer" and "inner" sides clearly identifiable. All but the innermost embryonic envelope (EE4) are shed by the embryo about 6 h before hatching. Permeability of the EC during the 12-d incubation period is found to decrease between 0 and 5 d after oviposition, and then increase until hatching. Fluorescently labeled lectins react positively with the OIC, indicating the presence of glucose and *N*-acetylglucosamine residues. Thus, the palaemonid EC is a dynamic structure throughout embryonic development.

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Abbreviations: embryonic coat (EC); embryonic envelope (EE); outer investment coat (OIC); scanning electron microscopy (SEM); transmission electron microscopy (TEM); fluorescein isothiocyanate (FITC); Tris[hydroxymethyl]aminomethane (Tris).

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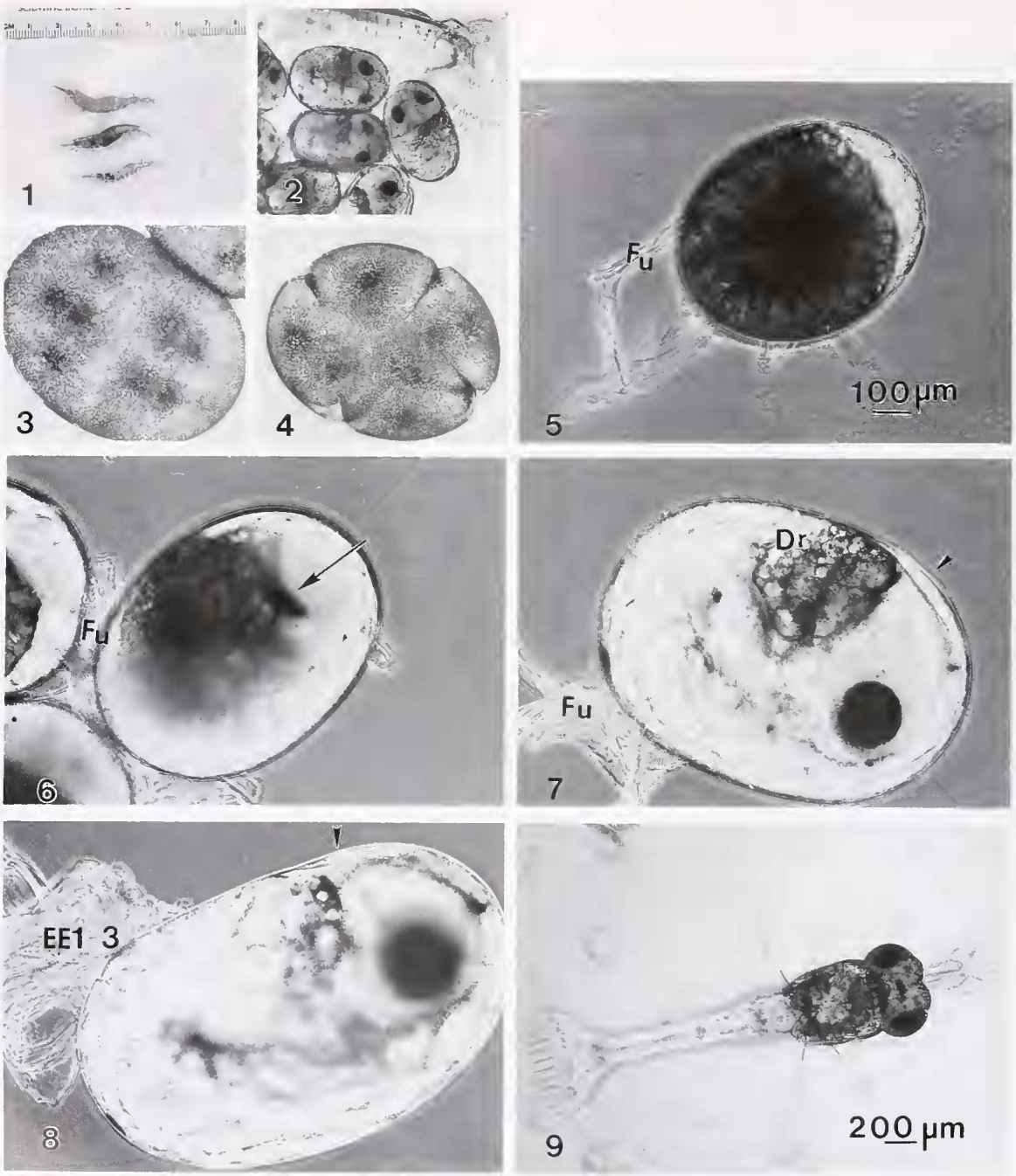
Introduction

Grass shrimp, *Palaemonetes pugio,* inhabit estuaries in the coastal regions of the eastern United States. During mating, the male places a spermatophore on the thorax of a mature female, near the opening of the gonopores. As eggs are extruded from the oviduct, they pass across the spermatophore and are fertilized externally. Eggs are deposited, or oviposited, on setae of the pleopods of the female. After about 2 weeks of incubation, the embryos hatch as zoeal larvae (Broad, 1957). During this time, the embryonic coat (EC) must protect the embryos from microbial, physical, and possibly chemical conditions of the ambient water while allowing passage of gases and other metabolites.

Various nomenclature has been used to describe the embryonic coat in marine decapods. Lobsters have a fertilization envelope or chorion (Talbot, 1981; Talbot and Goudeau, 1988); crabs have an extra-cellular capsule or fertilization envelope (Goudeau and LaChaise, 1980a, b; Goudeau and Becker, 1982), penaeid shrimp have a hatching envelope (Clark and Lynn, 1977; Pillai and Clark, 1987), and *Palaemon* has an extracellular capsule (Goudeau *et al.,* 1991). Investigations have revealed at least one additional "coat" besides the fertilization or hatching envelope as part of the protective covering of the developing embryo. Lynn *et al.* (1993) reported the presence of three or more "envelopes" during later stages of development in the penaeid shrimp *Sicyonia ingentis.* Goudeau and LaChaise (1983) and Goudeau *et al.*



Figures 1–9. Adult and embryonic stages of the grass shrimp, *Palaemonetes pugio.*

Figure 1. Photograph of *P. pugio* adults. Top, mature female; middle, ovigerous female; bottom, mature male.

Figure 2. Embryos 8 d after oviposition are shown attached to the ventral abdomen of a female. The embryos were attached to setae on the female pleopod by a cement that also forms the outer investment coat (OIC) of the embryonic coat.

Figure 3. Phase contrast micrograph of fertilized eggs on Day 0 within 4 h of ovipositioning. The embryo had progressed through karyokinetic divisions without cytokinesis.

Figure 4. Cytokinesis began after the embryos had completed three karyokinetic divisions.

Figure 5. The tissue cap stage was seen 3 d after oviposition. The tissue cap (clear area at the animal pole) is the developing embryo. The attachment of the egg by the funiculus, Fu, may provide spatial orientation for embryo development.

Table I

Development of Palaemonetes pugio *at 27°C and 20‰ salinity*

Embryonic stage	Day of development	Diameter (mm)	Standard deviation
Oviposition/karyokinesis/ cytokinesis	0	0.59	0.026
Late cleavage/early gastrulation	1	0.62	0.021
Gastrulation	2	0.67	0.028
Tissue cap	3	0.66	0.027
Cephalothorax delineation	4	0.66	0.021
Heartbeat initiation	5	0.69	0.020
Eye pigmentation	6	0.72	0.016
Embryonic eye	7	0.76	0.021
Early compound eye	8	0.80	0.019
Completed compound eye	9	0.84	0.048
Protozoeal embryo	10	0.84	0.030
Protozoeal embryo	11	0.82	0.047
Protozoeal embryo/pre-hatch	12	0.98	0.030
Hatch—1st zoeal larva	12	2.28	0.340

For each stage, the diameter is the mean value for 24 embryos measured across the longer axis of the oval-shaped egg. The hatch length is the "unfolded" length of the zoeal stage larva. Days of development represent 24-h periods at 27°C and 20‰ salinity.

(1990) reported five "coats" in the brachyuran crab *Carcinus maenas* and six "coatings" in the lobster *Homarus gammarus*. Morphological studies of the EC in palaemonid shrimp have been published (Sandifer and Lynn, 1980; Fisher and Clark, 1983; Lynn and Clark, 1983; Goudeau *et al.*, 1991), but none of these studies have examined embryos beyond the first few hours after oviposition.

On the basis of observations of development in *Palaemonetes pugio,* we have adapted terminologies from Talbot and Goudeau (1988) for the lobster and from Lynn *et al.* (1993) for penaeid shrimp. Crustacean decapods, in general, have an embryonic coat (EC) surrounding the developing embryo. This coat comprises one or more embryonic envelopes (EE) plus, in animals that brood their young, an outer investment coat (OIC) or cement that attaches the embryos to the female. Each EE, in turn, may be formed of several different layers. Thus, EEs are defined as layers that generally appear together as a single unit. The layers are morphologically distinct regions of the envelope. The origin of the layers that form the EE is usually embryonic, whereas the OIC origin may be maternal, embryonic, or both. In this study, we show that the EC is morphologically and functionally dynamic throughout embryogenesis.

Materials and Methods

Grass shrimp, *Palaemonetes pugio,* were collected from waters in east Escambia Bay, Pensacola, Florida, during the summer and fall of 1995. Adults were maintained at 24°C temperature and 20‰ salinity in flow-through aquaria. Animals were held in these conditions for 2 weeks before ovigerous females were removed. This period ensured that eggs removed from the females were oviposited under laboratory conditions. Embryos were gently removed from females and their ages were determined by using developmental stages as indications of approximate times postextrusion, *i.e.*, after oviposition (Tyler-Schroeder, 1978). A method modified from Fisher and Foss (1993) was used to culture the embryos. Each embryo was placed in a separate well of a 24-well plastic tissue culture plate. The plates were incubated at 27°C and 20‰ salinity with continual agitation at 60 rpm on a rotary shaker. Under these conditions, hatching was consistently 11–13 d after oviposition, with most embryos hatching after 12 d (Fisher and Foss, 1993).

Embryo development

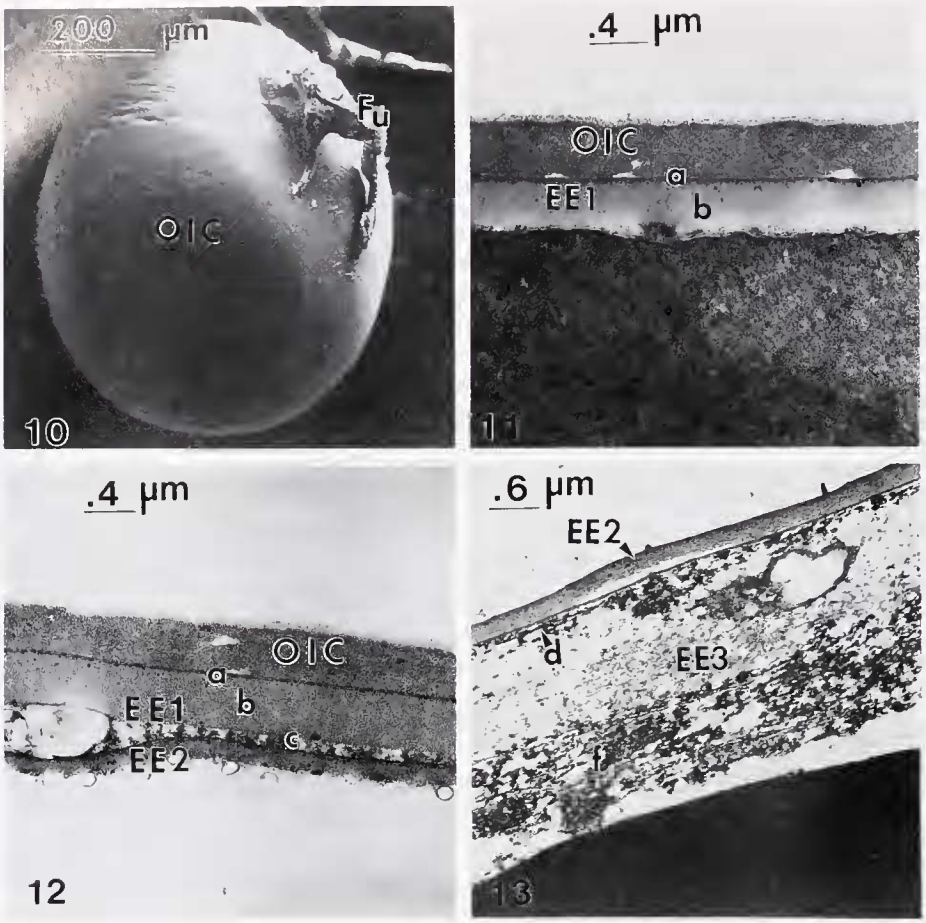
Embryos at different developmental stages were removed from culture plates, placed on glass slides in seawater in a ring of petroleum jelly, covered with glass coverslips, and photographed using phase microscopy on an inverted microscope. Embryo lengths were obtained using a Microcomp particle analysis system attached to a video capture system mounted on a dissecting microscope. Twenty-four embryos were measured daily throughout the course of development. Mean embryo lengths and standard deviations were calculated for each day of development.

Figure 6. At 7 d after oviposition, eye development (arrow) was well advanced. The cephalothorax and abdominal regions were well defined. Fu, funiculus.

Figure 7. By 10 d after oviposition, eye formation appeared complete. The telson (arrowhead) was wrapped around the cephalothorax of the embryo. Lipid droplets (Dr) were seen in the region of the developing hepatopancreas. Fu, funiculus.

Figure 8. At 12 d after oviposition, about 6 h before hatch, EE1–EE3 were shed (EE1–3), remaining attached only at the point of the funiculus. Only EE4 remained around the embryo. Arrowhead indicates the telson still within EE4. Figures 3–8 are presented at the same magnification to demonstrate the increase in embryo size during development. Bar equals 100 μm.

Figure 9. The first zoeal larvae hatched 12 d after oviposition. Bar equals 200 μm.



Figures 10–19. Electron micrographs of *Palaemonetes pugio* embryos showing the development of the embryonic coat.

Figure 10. A scanning electron micrograph of an embryo at 3 d after oviposition shows the smooth outer surface of the outer investment coat (OIC) with the funiculus, Fu. The funiculus was the point of attachment of the embryo to a seta of the female pleopod.

Figure 11. At 3 d after oviposition, the OIC and first embryonic envelope (EE1) were the only parts of the EC seen with TEM. EE1 had two layers, a thin, electron-dense layer, "a," and a thicker, less electron-dense layer, "b."

Figure 12. By 5 d after oviposition, EE1 had 3 layers, "a" and "b" as defined above, and layer "c," a flocculent, loosely defined layer. A second envelope, EE2, had formed and, in some areas, elevated to just below EE1.

Figure 13. In a section of another embryo 5 d after oviposition, EE2 (arrow) remained close to the embryo. The third envelope, EE3, was seen forming internally to EE2 and had a thin, electron-dense outer layer (d) and a looser, inner fibrillar layer (f).

Embryonic coat development

Morphological changes in the embryonic coat (EC) were examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Embryos for SEM and TEM were fixed for 24 h in 1.6% formaldehyde and 0.8% glutaraldehyde in seawater. Alternatively, embryos were fixed for 4 h in 3% glutaraldehyde, then 24 h in Bouin's fixative. Samples were washed three times with 0.2 *M* phosphate buffer and post-fixed in 1% osmium tetroxide in phosphate buffer, then washed in distilled water. SEM samples were then frozen in liquid nitrogen, loaded into the Zeiss DSM 962 scanning electron microscope, sublimated, then sputter-coated with palladium-gold. For freeze-fracture samples, the embryos were fractured before sublimation. Samples for TEM analysis were dehydrated with acetone, infiltrated with Spurr's resin, and then embedded in fresh resin. Thin sections were cut, double stained with lead citrate (Venable and Coggeshall, 1965) and aqueous uranyl acetate, and observed with TEM.

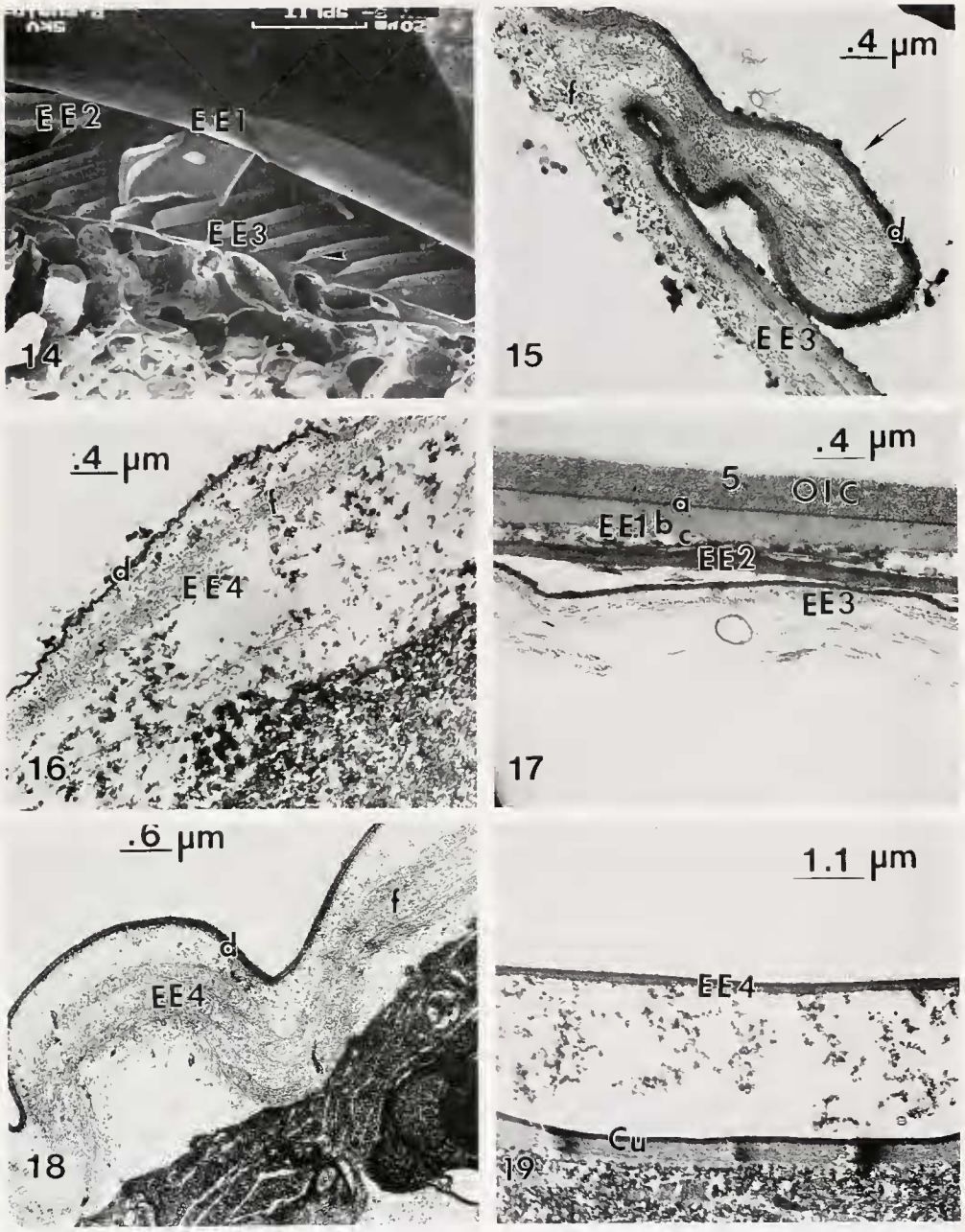


Figure 14. Between 5 and 7 d after oviposition, multiple envelopes of the EC had formed and are visible in this SEM of a freeze-fractured 7-d embryo. Three of the envelopes, EE1, EE2, and EE3, were seen, with EE3 having an elaborate system of ridges (arrowhead).

Figure 15. A cross-section of a ridge (arrow) in EE3 was seen in TEM from a 7-d embryo. The dense layer (d) and fibrillar layer (f) of EE3 were better organized than in earlier micrographs.

Figure 16. By 7 d after oviposition, a fourth envelope had formed interiorly to EE3. This envelope, EE4, remained close to the embryo and developed a thin, electron-dense outer bilayer (d) and a fibrillar inner layer (f).

Figure 17. By 10 d after oviposition, four EE surrounded the embryo, with EE1–EE3 found juxtaposed and relatively distant from EE4 and the embryo. This micrograph of the EC at 10 d shows all the layers of EE1 (a, b, and c), as well as EE2 and the two layers of EE3.

Figure 18. At 10 d after oviposition, EE4 was still found close to the embryo. The dense outer layer (d) had a bilaminar, railroad-track appearance, while the inner fibrillar layer (f) had become more organized. EE4 formed large folds with a wavelike appearance.

Figure 19. Before hatching from EE4, the embryo formed the cuticle (Cu) of the exoskeleton. This cuticle had a trilaminar appearance in contrast to the bilaminar appearance of EE4. The characteristic layering of the cuticle had already begun.

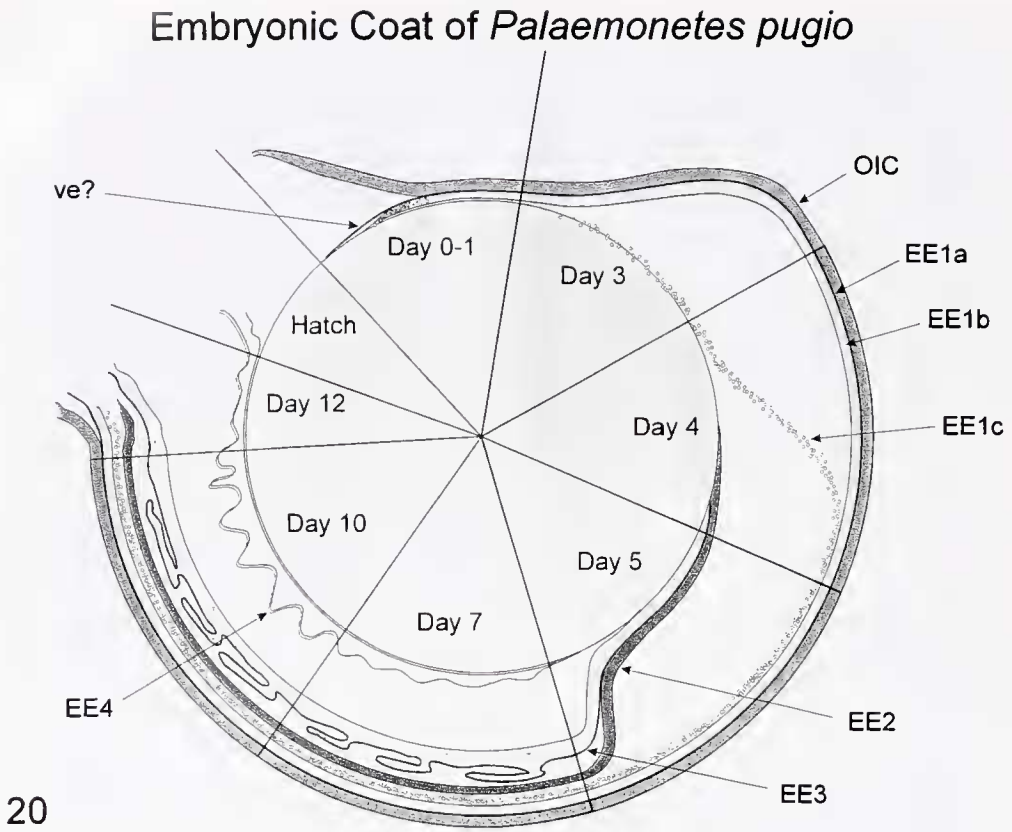


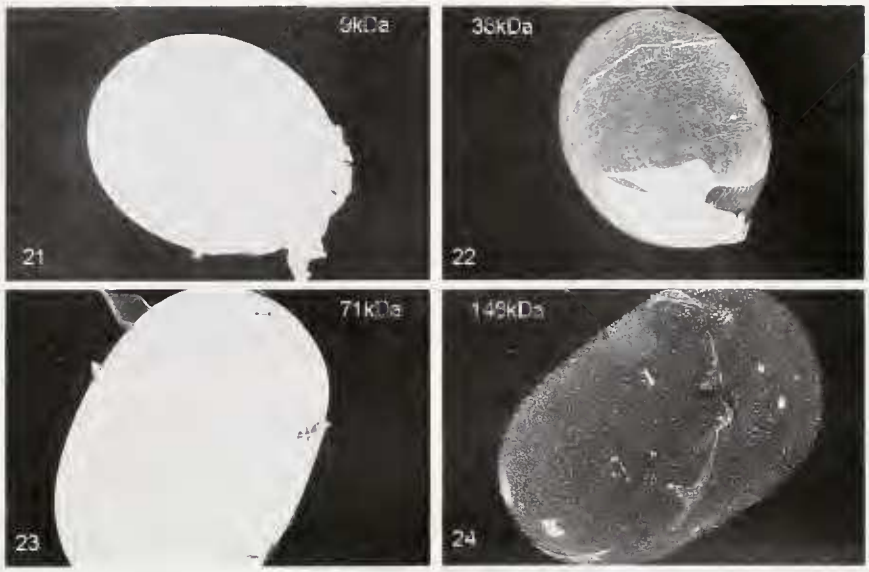
Figure 20. A schematic presentation of the sequence of formation of the embryonic coat of *Palaemonetes pugio*, including the embryonic envelopes (EE) and layers. ve, vitelline envelope; OIC, outer investment coat; $EE1_a$, $EE1_b$, and $EE1_c$, the three layers of the first embryonic envelope; EE2, EE3, EE4, the second, third and fourth embryonic envelopes, respectively. The layers and distance from the embryo are not to scale.

Embryonic coat permeability

Permeability assays were performed as reported by Glas *et al.* (1995) and Legge (1995). Embryos at various stages of development were incubated with fluorescein isothiocyanate (FITC)-labeled dextrans of five molecular weights (Sigma Chemical Co.) by adding 10 μl of fluorescently labeled dextran (stock = 50 mg/ml in distilled water) to individual wells containing embryos and seawater at 20‰ salinity. After 30 min incubation in the dark at 27°C, embryos were rinsed three times with filtered seawater and examined for dextran penetration beside the EC using 488 nm filters on an inverted microscope. At least four embryos were observed for each concentration of dextran. Penetration of the EC by the dextran was determined by focusing through the embryo. For illustration, embryos were examined and photographed using confocal microscopy. The embryos were scanned using the same settings for laser intensity (488 nm blue laser), offset and gain.

Lectin-binding affinity

Embryos with intact ECs at 8 d and 12 d after oviposition were incubated with FITC-labeled lectins. The lectins (and their affinities for oligosaccharides) used in this study were Concanavalin A agglutinin (Con A), α-D-glucosyl residues, especially glucose and *N*-acetylglucosamine; *Limulus polyphemus* bacterial agglutinin (LPA), *N*-acetylated D-hexosamines, especially D-glucuronic acid and *N*-acetylneuraminic acid; and *Triticum vulgaris* agglutinin (wheat germ agglutinin, WGA), *N*-acetyl-β-D-glucosaminyl residues, especially *N*-acetyl-β-D-glucosamine oligomers. Lectin stock solutions, 1 mg/ml for LPA and WGA and 2 mg/ml for Con A, in 0.05 *M* Tris (pH 7.2), with 0.01 *M* calcium, magnesium, and manganese chlorides as trace metals, were made according to the procedures of Kiernan (1990); 10 μl of stock solution was added to individual wells containing embryos in 1.0 ml seawater at 20‰ salinity. The embryos were incubated in the dark for 1 h at 27°C, then rinsed three times



Figures 21–24. Permeability of the embryonic coat (EC) of *Palaemonetes pugio* was determined using FITC-labeled dextrans. Embryos were viewed using confocal microscopy.

Figure 21. At 3 d after oviposition, the embryo EC was permeable to FITC-labeled dextrans weighing 9 kDa. The dextran inside the EC showed the entire embryo as a bright image.

Figure 22. An example of an embryo 3 d after oviposition incubated with dextrans weighing 38 kDa. The dextran does not remain in the inside of the EC so the outline of the embryo is visible.

Figure 23. Embryo EC was permeable to the FITC-labeled dextran weighing 72 kDa at 10 d after oviposition, so the embryo appears as a bright oval.

Figure 24. Embryo EC was impermeable to the dextran weighing 148 kDa at 10 d after oviposition.

with seawater. The embryos were then observed for fluorescence on the outside of the OIC. To block for nonspecific binding, embryos were pretreated for 30 min with 1 mg/ml bovine serum albumin in the Tris buffer, then rinsed with seawater. As sugar controls, lectins were incubated with 1 *M* solutions (0.3 *M* for *N*-acetylneuraminic acid) of specific oligosaccharides before being added to the wells (Kiernan, 1990). Sugars used were sucrose, glucose, and *N*-acetylglucosamine (for Con A); D-glucuronic acid and *N*-acetylneuraminic acid (for LPA); and *N*-acetylglucosamine and *N*-acetylneuraminic acid (for WGA).

Table II

Permeability of the embryonic coat of Palaemonetes pugio *to FITC-labeled dextrans*

Day of development	FITC-labeled dextran molecular weight (kDa)					
	4.3	9.3	19.6	38.9	71.2	148.3
3	+	+	+	±	−	−
4	+	+	−	−	−	−
5	+	+	−	−	−	−
7	+	+	+	±	−	−
10	+	+	+	+	+	−
12	+	+	+	+	+	+

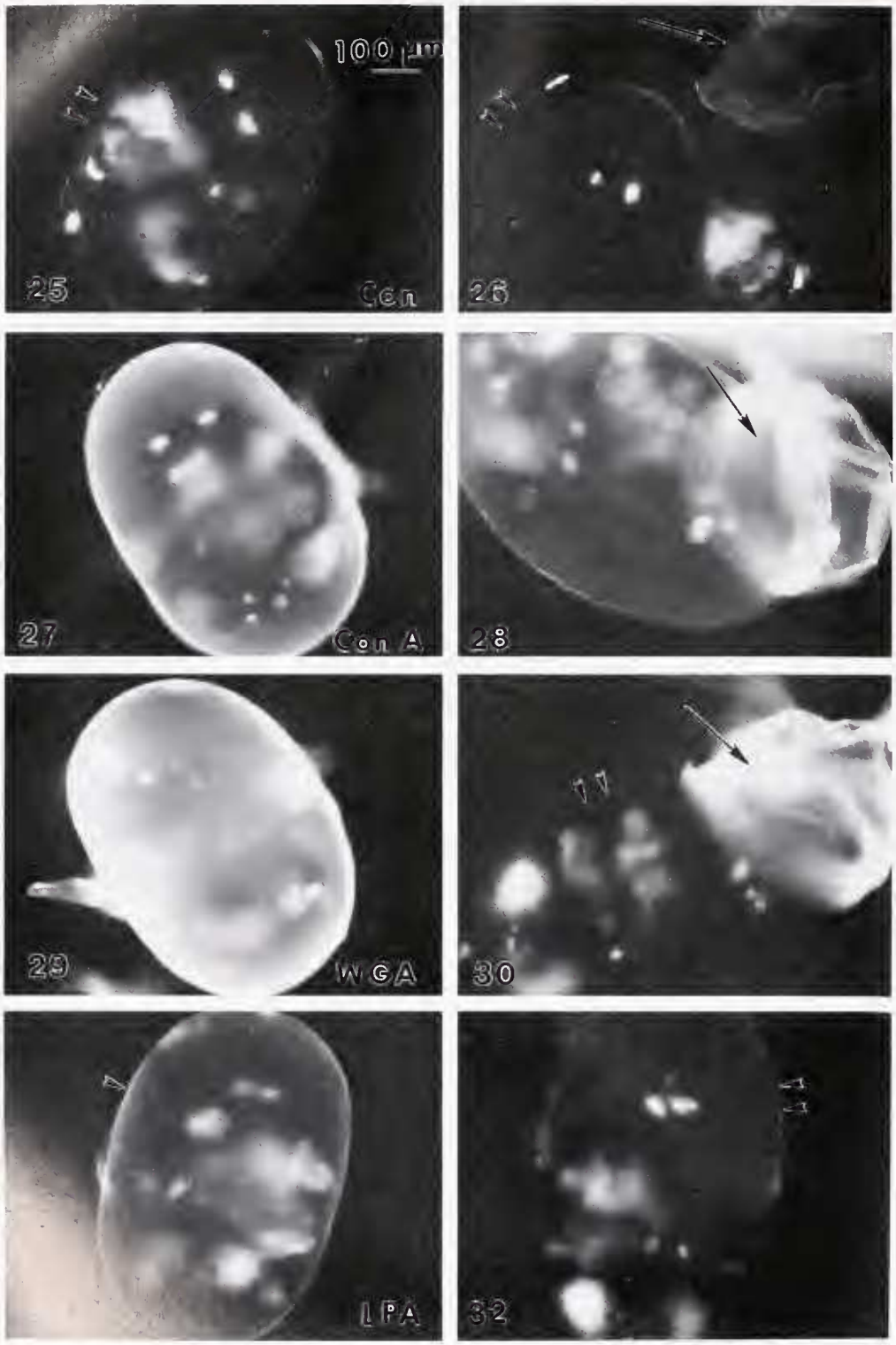
Permeability is recorded as + (permeable) or − (nonpermeable) on the basis of the presence or absence of fluorescent dye in the perivitelline space; ± indicates that fluorescence was found in the perivitelline space of 50% of the eggs. Each symbol represents the observation of at least four embryos.

Results

Embryo development

Development and hatching of *P. pugio* closely followed the sequence of events described by Broad (1957), Davis (1965), and Thomas (1970). Ova of *P. pugio* were released from the female oviduct and passed over the male spermatophore, fertilized, and attached to the female pleopods during oviposition (Figs. 1 and 2). At the time of attachment, the zygotes were single celled, carrying both male and female pronuclei. After pronuclear fusion, the zygote proceeded through three karyokinetic divisions (Fig. 3) before cytokinesis (Fig. 4). The orientation of the embryo within the EC remained the same, with the funiculus, or attachment stalk, at the distal edge of the presumptive cephalothorax.

By 3 d, or 72 h after oviposition, embryos reached the tissue cap stage as described by Tyler-Shroeder (1978): the clear region was at the animal end of the embryo, and yolk filled much of the remainder of the egg (Fig. 5). Af-



Figures 25–32. Embryos at 8 d and 12 d after oviposition were exposed to FITC-labeled lectins.

Figure 25. Control embryos, Con, at 8 d after oviposition had some autofluorescence, especially in the eyes, yolk, and other pigmented areas. Arrowheads indicate the outline of the embryo.

Figure 26. After the outer EE1–EE3 were shed (arrow), the remaining inner coat (arrowheads) also did not show any fluorescence.

ter 5 d of development, the heart had begun beating, and segmentation was apparent as the embryos were elongating. Eye pigmentation was evident by 7 d after oviposition (Fig. 6), with development continuing until 9 d, when the compound eye was fully formed. At this time, the embryos appeared to be completely formed externally. At 10 d (Fig. 7), lipid "droplets" were visible in the region destined to become the hepatopancreas, and the amount of dark yolk had greatly diminished.

The size of *P. pugio* embryos cultured at 27°C and 20‰ salinity increased throughout development (Table I). After 12 d of development and about 6 h before hatching, the embryos shed the outer portion of the EC (Fig. 8) and rapidly increased in size from 0.82 mm to 0.98 mm within 24 h (Table I). The embryos finally broke through the remaining envelope to hatch as first zoeal larvae (Fig. 9). Development was consistent with that described by Fisher and Foss (1993) so that hatching consistently occurred after 11–13 d of incubation.

Embryonic coat formation

During oviposition, the female released a "cement" that coated the newly fertilized eggs in a sticky layer for attachment to setae on the pleopods (Figs. 2 and 10). By 4 h after oviposition, the cement had formed an outer investment coat (OIC) that surrounded the embryo (Fig. 10) and also comprised the attachment strands, or funiculi, which connected the embryos to the pleopods. The embryos were always attached in the same relative position; *i.e.,* the funiculus was always at the position that became the posterior end of the carapace on the dorsal side (see Davis, 1965). The funiculus and attachment points to other embryos were continuous with the OIC surface. Externally, the OIC had no distinguishing features other than the funiculus attachments (Fig. 10). The smooth exterior surface of the embryos sometimes had bacterial growth during later stages of embryonic development, a common occurrence for externally brooded decapod embryos (Fisher, 1983). Otherwise, no dramatic external changes occurred until the outer envelopes were shed. The stickiness of the cement was no longer noticeable by 3 d after oviposition as the eggs no longer clumped together on contact.

Four hours after oviposition, the embryo was surrounded by the OIC and a bilayered embryonic envelope, EE1. Transmission electron micrographs revealed that the OIC comprised the outermost layer of the coat surrounding the embryo and was about 0.4 μm thick (Fig. 11). The first EE initially consisted of a thin, outer layer, $EE1_a$, and a thick, inner layer, $EE1_b$. The thickness of the $EE1_b$ had increased to 0.38–0.4 μm by 3 d after oviposition (Fig. 11), after which no further increase was seen. Within 3–5 d after oviposition, a third layer, $EE1_c$, was added to the previous two layers. This layer was composed of an electron-dense, filamentous material with a loosely organized structure (Fig. 12). This layer did not always stain well, appearing as a sparsely filled space between EE1 and EE2 after its formation. Usually EE1 was closely associated with the OIC.

The second envelope, EE2, began to appear late in the 4th d after oviposition. At 5 d after oviposition, EE2 elevated to remain closely associated with EE1 (Fig. 12). In a section of another 5-d embryo, EE2, which had not yet elevated could be seen as a dense envelope outside the newly forming EE3 (Fig. 13). When stained for electron microscopy, EE2 measured about 0.12–0.2 μm, with a thin, dense outer layer and a less dense inner layer.

A third envelope, EE3, appeared by 5 d after oviposition. This envelope consisted of an electron-dense, thin outer layer and a wide band of heterogeneous electron-dense and lucent regions (Fig. 13). By 7 d, this envelope had condensed and formed ridges in some parts of the embryo (Fig. 14). The ridges did not circumscribe the embryo. In section, a ridge was seen as an area where EE3 had folded back on itself (Fig. 15). The condensed EE3 had an outer electron-dense layer about 0.13 μm thick and a fibrillar, less dense inner layer about 0.26 μm

Figure 27. The lectin Concanavalin A (Con A) had a high affinity for the outer investment coat (OIC) of the 8-d embryo.

Figure 28. After the outer EEs were shed immediately prior to hatching, the Con A lectin had only a slight affinity for the remaining envelope, EE4. The discarded EE1–EE3 and OIC (arrow) still reacted strongly with the lectin.

Figure 29. The wheat germ agglutinin lectin, WGA, had a very strong affinity for the OIC of the 8-d embryo.

Figure 30. At 12 d after oviposition, after the outer EE1–EE3 and OIC were shed, the WGA lectin had no affinity for EE4, which remained around the embryo (arrowheads). The discarded EE1–EE3 and OIC (arrow) still reacted to the lectin.

Figure 31. *Limulus polyphemus* agglutinin showed only low nonspecific affinity for the OIC of the 8-d embryo (arrowhead).

Figure 32. At 12 d after oviposition, after EE1–EE3 and the OIC were shed immediately before hatching, the LPA lectin had no affinity for EE4 (arrowheads), which remained around the embryo. Bar equals 100 μm.

thick (Fig. 15). The fibrils tended to lay parallel to the embryo surface. By 10 d after oviposition, this envelope was about 0.5 μm wide and, in regions where EE3 had not formed ridges, was found subjacent to EE2 (Fig. 17).

The final envelope, EE4, was first seen at 7 d after oviposition. The morphology of EE4 was initially similar to EE3 during formation in that the outer layer was electron-dense and the inner layer was fibrillar and irregular (Fig. 16). The layers were near the embryo surface and measured about 0.5 μm thick. By this time, EE3 had moved away from the embryo toward the outer envelopes that were layered beneath the OIC (Fig. 17). At 10 d after oviposition, EE4 had large wave-shaped folds (Fig. 18) and measured about 1.2 μm thick. The outer layer of EE4 had formed a bilaminar "railroad-track" appearance, and the inner fibrillar layer was oriented parallel to the embryo surface. The developing embryo was seen immediately below the waves of EE4 (Fig. 18). EE4 remained near the embryo surface throughout the rest of embryonic development.

Six hours before hatching, the OIC and the outer envelopes, EE1–EE3, were shed, remaining attached to the embryo only at a point on the posterior dorsal edge of the cephalothorax (Davis, 1965). The innermost envelope, EE4, no longer had folds in it, but was found close to the embryo (Fig. 19). The thickness of the envelope was greatly reduced, as though stretched. The fibrils were scattered throughout the space between the dense outer layer and the embryo. At this time, the cuticular layer of the embryonic exoskeleton had formed (Fig. 19). The cuticular layer could be distinguished from EE4 by the morphology of the dense outer layer. In EE4, this layer was bilaminar, or "railroad track," in appearance, whereas the dense layer of the forming cuticle had a trilaminar appearance.

The sequence of events for the formation of the extraembryonic coat is summarized in Figure 20. The relative widths of the embryo, envelopes, layers, and perivitelline space are not drawn to scale. The origin of EE1 from a precursor layer or "vitelline envelope" is speculative at this time.

FITC-labeled dextrans indicate permeability change with time

When embryos were incubated with FITC-labeled dextrans 3 d after oviposition, only those dextrans weighing less than 39 kDa were able to penetrate the EC (Figs. 21 and 22). Permeability became more restrictive by 7 d after oviposition, when only the dextrans weighing less than 19 kDa could penetrate the EC. This restriction continued until 7 d after oviposition, when larger size dextrans were able to penetrate the EC. As the embryos aged further, the permeability to dextrans increased, with only the largest dextran (148 kDa) being excluded from the EC by 10 d after oviposition (Figs. 23 and 24). Immediately prior to hatching, 12 d after oviposition, all dextrans tested penetrated the inner envelope, EE4 (see Table II).

Lectin specificity indicates β-glucoside saccharides

The OICs of embryos were exposed to three lectins—Concanavalin A (Con A), *Limulus polyphemus* agglutinin (LPA), and wheat germ agglutinin (WGA)—at 8 d and 12 d after oviposition to test for the presence of specific terminal oligosaccharides (Figs. 25–32). Embryos without any lectins added showed slight autofluorescence in pigmented regions (see Thomas, 1970, for description) but no fluorescence in the EC (Figs. 25 and 26). The OICs of embryos incubated with Con A 8 d after oviposition gave a positive fluorescent reaction (Fig. 27), indicating terminal sucrose, D-glucosyl and *N*-acetyl-D-glucosamine residues. After the EEs were shed at 12 d after oviposition, there was little fluorescent reaction with EE4 (Fig. 28), but the discarded EEs and OICs still reacted strongly. At 8 d after oviposition, the OIC had a strong fluorescent reaction with WGA (Fig. 29), indicating terminal *N*-acetyl-D-glucosaminyl residues as well as β-*N*-acetylglucosamine oligomers. Embryos 12 d after oviposition had little fluorescent reaction with EE4 when treated with WGA (Fig. 30), although the discarded EEs and OICs still reacted. At 8 d after oviposition, LPA showed only nonspecific binding with the OIC (Fig. 31). At 12 d after oviposition, when the outer EE and OIC were shed, EE4 showed no affinity for the LPA lectin (Fig. 32). When blocked for nonspecific binding using bovine serum albumin, the Con A and WGA lectins continued to react strongly with the OIC, but LPA lectin showed no reaction with the OIC. When each lectin was preincubated with the corresponding sugars, fluorescence was blocked in all treatments (data not shown).

Discussion

The formation of the EC within the first four hours after oviposition has been documented in the prawn *Palaemon serratus* by Goudeau *et al.* (1991) and in *Macrobrachium* by Sandifer and Lynn (1980) and by Lynn and Clark (1983). The formation of additional coats after the EC has formed has also been reported in other species such as penaeid shrimp (Lynn *et al.*, 1993) and lobster (Talbot and Goudeau, 1988). What has not been described in previous studies is that formation of the EC may continue, as in *P. pugio*, for up to 10 d or so after oviposition. In *P. pugio* during this time, at least three additional EEs are formed around the developing embryo that are not present at 3 d after oviposition. These envelopes show distinct morphological character-

istics that allow each to be identified during development. The EEs form and mature morphologically for 10 d after oviposition of the embryos. Together, the OIC and all the EEs form the protective EC of the palaemonid shrimp. At 12 d after oviposition, the outer envelopes, EE1–EE3, are shed; only the innermost envelope, EE4, remains around the embryo. Hatching occurs when the embryo emerges from EE4. The hatching of *P. pugio* is described by Davis (1965). However, the role of the funiculus in the development of the embryo has not been closely examined.

Broad (1957) states that a prezoeal molt takes place before hatching. We saw no evidence of a molt that, as Helluy and Beltz (1991) have seen in lobster, conforms to the surface of the embryo before hatching. The cuticle layer seen at 12 d conformed to the surface of the embryo, but was clearly separate from EE4. Although the innermost envelope, EE4, was morphologically similar to the adult crustacean exoskeleton, there were distinct morphological differences when compared to the embryonic exoskeleton (compare Fig. 18 and Fig. 19).

Changes in permeability of the EC to labeled dextrans coincide with the formation of the envelopes. As EEs were added, the permeability of the EC decreased. After all the envelopes had formed and the embryo had developed further, the permeability increased. The permeability was greatest as the embryo approached hatching; *i.e.*, after the OIC and EE1–EE3 had been shed and only EE4 remained around the embryo. Some of these results may explain in part the difficulty in embryo fixation reported by Thomas (1970) and also seen in this laboratory.

Lectin affinity studies lend some insight into the composition of the EC. The lectins are too large in molecular weight to penetrate the EC. Only Con A may be small enough to pass in and out of the EC; however, it appears to bind to the EC, as indicated by the strong fluorescent image. The strong Con A and WGA lectin affinity indicates the presence of a terminal glucosyl and *N*-acetylglucosamine residues. Contrast the binding of lectin to the OIC with the lack of binding to EE4 after the outer coat has been shed.

The results of this study are important to those using *P. pugio* for bioassays to understand the effects of toxicants and microorganisms on the developing embryo. The changing morphological and functional aspects of the EC throughout the development of the embryo profoundly affect the stage-specific sensitivity and susceptibility of the embryo. Further research is needed to address the importance of the OIC and funiculus in successful development, the functional roles of the different layers of each EE, and the effects of physical and chemical stressors on the progression of EC formation.

In this paper, we propose terminology to describe a complex protective structure. We use terms that are descriptive yet familiar, avoiding commonly used terms such as "extracellular coat" because we feel they are misleading. Because we are studying the envelopes around an embryo with multicellular organization, not just the newly fertilized zygote, the word "extracellular" could be misleading to those unfamiliar with the historical usage. We use "coat" to encompass the entire protective structure, since normally we think of only one coat being used at a time. We have continued to use "envelope" since it is now widely accepted in several species. Ideally, this usage will further promote the standardization of terminology among developmental biologists.

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